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Toshikazu NAKAMURA

Examiner: Aaron J. Kosar

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DECLARATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Sir:

I, Satoshi Takeo, declare that:

I was born in Nagasaki, Japan, in 1942;

I am a citizen of Japan and a resident of 1-25-42-802, Momochi, Sawara-ku, Fukuoka, 8140006, Japan;

I received my doctor degree on the study of "Effects of isoproterenol on myocardial fatty acid metabolism" at Kumamoto University Medical School, Kumamoto, Japan, in 1977;

I have worked as Scientist of the Institute of Organic Chemistry at Tanabe Seiyaku Co. Ltd. in Japan from 1967 till 1973;

I was a postdoctoral fellow of Canadian Heart Foundation of Department of Physiology of Faculty of Medicine at the University of Manitoba, Canada, from 1977 till 1979;

I was a visiting Scientist of the Medical and Biological Institute at Casella AG, Germany, from 1979 till 1980.

I was appointed as Assistant Professor of Department of Kumamoto University Medical School, Kumamoto Japan, in 1980;

I was appointed as Assistant Professor of Faculty of Medicine at Ryukyu University, Okinawa Japan, in 1982;

I was appointed as Professor of Faculty of Pharmaceutical Sciences at Fukuyama University, Hiroshima Japan, in 1983;

I was appointed as Professor of Division of Pharmacology at Tokyo University of Pharmacy and Life Sciences, Tokyo Japan, in 1989;

I am one of the inventors for this application.

I have many reports (43) relating to microsphere embolism-induced, sustained ischemia in brain, in corroboration with my colleagues, as shown below. I believe that we are experts for the pathogenesis of microsphere-induced sustained ischemia and drug effects on it. The reports are as follows:

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Commun, 368(1):151-6, 2008.

The experiment set out below was conducted under my supervision.

Experiment

Effects of human recombinant HGF treatment on cerebral embolism-induced hyperpermeability in vascular vessel

1. Purpose

Cerebral capillaries are impaired by embolic substances, resulting in a progressive alteration in the permeability of the blood-brain barrier (BBB), and leading to formation of ionic edema, vasogenic edema and hemorrhagic conversion. When capillaries that form the BBB can no longer retain intravascular constituents, such as Na^+ , water, and serum proteins, and blood, these substances enter into the extracellular space of the brain and causes edema (Simard et al., *Lancet Neurol*, 6:258-268, 2007). The present study was designed to determine whether treatment with human recombinant HGF is capable of preventing the brain from cerebral embolism-induced alteration in vascular permeability and the following damage to cognitive function.

2. Experimental Animal

Wistar male rats each weighing 220 to 250 g were used as an experimental animal. The animals were freely given feed and water and acclimated in an artificial environment at a constant temperature ($23 \pm 1^\circ\text{C}$), constant humidity ($55 \pm 5\%$) and illumination for a predetermined time (a cycle of 12 hours of light and 12 hours

of darkness) in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals, and the Guideline for Experimental Animal Care, issued by the Prime Minister's Office of Japan.

3. Sample

Human recombinant DNA of hepatocyte growth factor (HGF) was prepared according to the known method (Nakamura et al., Nature 342:440-443, 1989) and used as a test sample.

4. Preparation of a model with microsphere-induced cerebral embolism

A model with microsphere-induced cerebral embolism was produced by the method described previously (Miyake et al., Stroke 24, 415-420, 1993) with some modification. Rats were anesthetized by intraperitoneal administration of 40 mg/kg sodium pentobarbital (Nembutal, Abbott Laboratories, Canada, USA), and then fixed in a supine position. After midline incision, the right common carotid artery was exposed, and the blood flow in the right external carotid and the right pterygopalatine arteries were temporarily ligated. A Seven-hundred of microsphere (diameter of 48 μ m, NEN-005, New England Nuclear Inc., Boston, USA) suspended in 20 w/v % dextran solution was injected into the right common carotid artery via a needle (25 G, TERUMO, Tokyo, Japan) connected to a polyethylene catheter (3 French size, Atom Medical Co., Ltd., Tokyo). After injection, the puncture wound was repaired with a surgical glue (Aron Alpha A, Sankyo Co., Ltd., Tokyo), and blood flow of the right external carotid and right pterygopalatine arteries was recirculated, and the surgical site was sutured. It took about 2 minutes to recirculate blood flow of the right external carotid and right pterygopalatine arteries. The same operation as described

above was carried out, and the rats to which 20 w/v % dextran solution only was injected was used as sham operation (Sham) rats.

The rats having cerebral embolism were divided at random into an HGF-administered group and a solvent (vehicle)-administered group. The continuous injection of HGF into the cerebral ventricle was conducted by administering a solution of HGF in physiological saline continuously for 7 days into the right cerebral ventricle via an osmotic pressure pump (Alzet model 2001, Alzet, Calif., USA) from 10 minutes after injection of the microspheres, whereby 30 µg HGF was administered per animal.

5. Statistical analysis

Statistical comparison among multiple groups was evaluated by analysis of valance (ANOVA) followed by *post-hoc* Fisher's protected least significant difference (PLSD) test. The escape latency of the water maze test was analyzed by using two-way ANOVA for repeated measures followed by *post-hoc* Fisher's test. Differences with a probability of 5% or less were considered significant ($P < 0.05$).

6. Changes in neurological deficits

At 15 hr after the operation for microsphere-induced cerebral embolism, the behavior of operated rats was scored based on paucity of movement, truncal curvature, and forced circling during locomotion, which are considered typical symptoms of stroke in rats. Each item was rated from 3 to 0 (3: very severe; 2: severe; 1: moderate; 0: little or none). The neurological deficits of the operated animals from day 2 to day 28 were determined at 10:00 AM every day.

Figure 1 shows the time course of changes in neurological deficits of sham-operated (Sham), microsphere-embolized (ME), and HGF-treated ME (HGF) rats.

About 84% of the animals with the operation for microsphere embolism showed stroke-like symptoms with a total score of 7-9 points, whereas the Sham animal did not reveal any neurological deficits throughout the experiment. Two-way ANOVA revealed no significant difference in changes in neurological deficits between HGF-untreated ME and HGF-treated ME groups. The neurological deficits of the ME animals disappeared on day 12 and thereafter. The results suggest that the neurological deficits seen in ME animals are not due to an injury in selective brain regions, but due to a postoperative outcome during the early period after microsphere embolism.

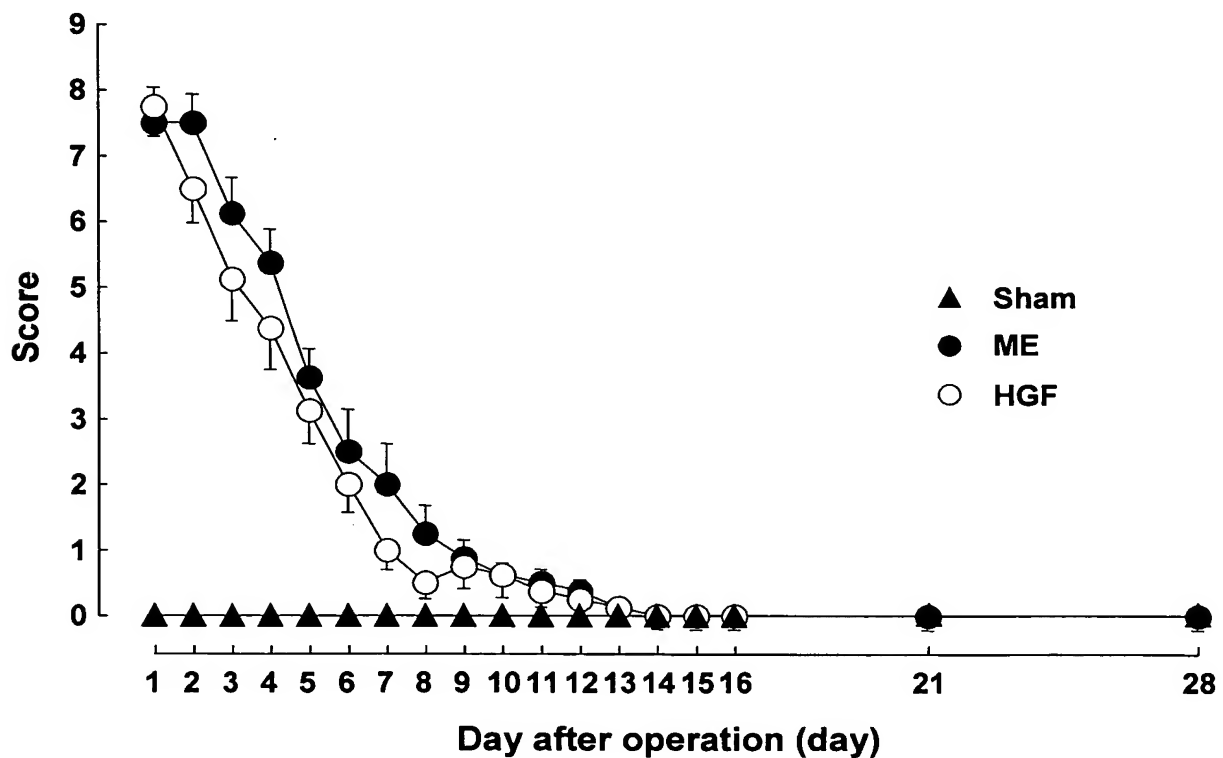


Fig. 1 Time course of changes in neurological deficits of the sham-operated (Sham, closed triangles), HGF-untreated ME (ME, closed circles), and 30 μ g/7days/animal HGF-treated ME (HGF; open circles) rats. Each value represents the mean \pm SEM of 8 animals. There was no significant difference in the neurological deficits between the ME and HGF rats.

7. Determination of leakage area of FITC-albumin

To determine the degree of disruption of BBB, we measured the leakage area of fluorescence isothiocyanate-conjugated albumin (FITC-albumin) according to the method of Cavaglia et al. (Brain Res 910:81-93, 2001). Rats were anesthetized with an intraperitoneal injection of 40 mg/kg pentobarbital. Bilateral carotid arteries of the animal were exposed and cannulated with polyethylene tubes (SP 31) attached to 24-gauge needles. FITC-albumin solution (10 mg/mL in 0.1 M phosphate buffered saline, pH 7.4), was perfused through the carotid arteries at a rate of 1mL/min (10 mL/kg) using a syringe pump. To avoid elevation of the cerebrovascular pressure, we withdrew the same amount of blood from the inferior vena cava. Immediately after perfusion, the rats were decapitated and their brains were isolated. The brains were fixed for 24 hr in 4% paraformaldehyde and then cryoprotected in a 10%, 20%, and 30% sucrose solution for 4 hr for each concentration. Then, the brains were embedded in Tissue-Tec O.C.T. compound (O.C.T. compound, Sakura, Tokyo) and frozen in liquid nitrogen. Frozen coronal brain sections (40 μ m thick) were cut on a cryostat, and thaw-mounted on poly-L-lysine-coated microslide glasses. Sections were embedded in 0.1 M phosphate buffered saline for 30 min, mounted with DAKO Fluorescent mounting medium (DAKO, USA), and thereafter observed under a fluorescence microscope. The leakage area of FITC-albumin (FITC-stained areas out of the cerebral vessels) was measured using an image analysis software.

Figure 2 shows the time course of changes in the leakage area of FITC-albumin in the selective regions to determine the disruption of BBB up to day 28 after the operation. Leakage of FITC-albumin started on day 1 and the maximal leakage was detected on day 3 after the embolism. Thereafter, the leakage area of FITC-albumin was decreased on days 5 and 7 after the embolism. The leakage area of

FITC-albumin was not detected on day 14 or 28 after the embolism. The results suggest that increased vascular permeability is induced during the early period after microsphere embolism, which may be involved in brain edema.

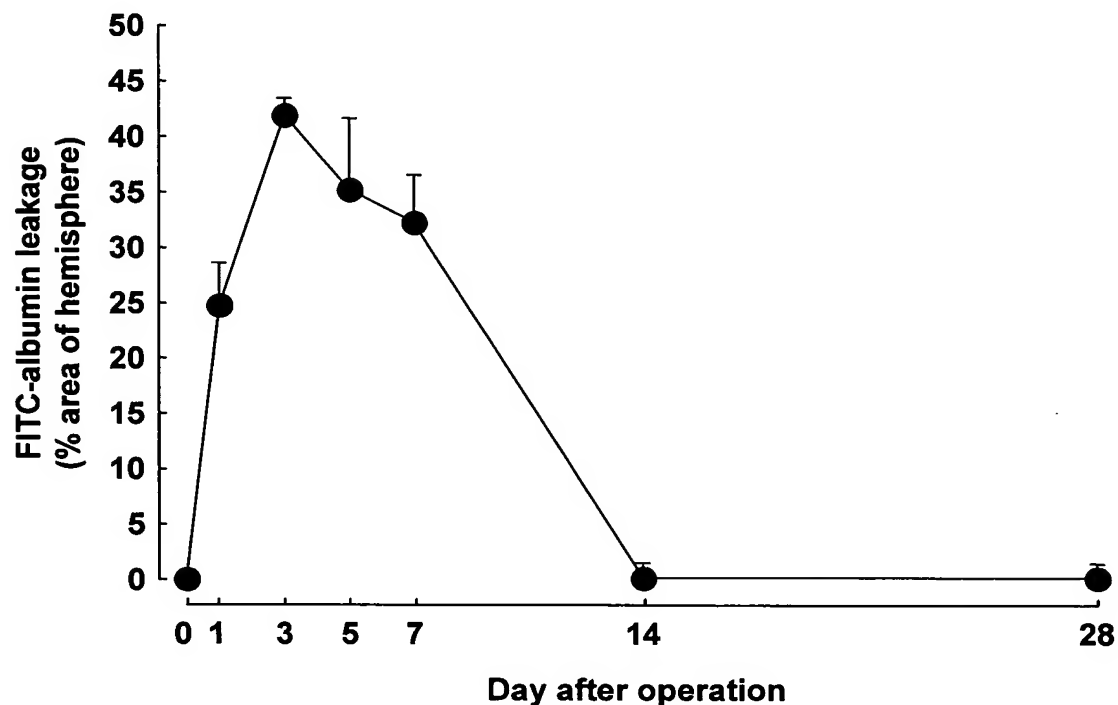


Fig. 2 Time course of changes in FITC-albumin leakage of the HGF-untreated ME rat on days 1, 3, 5, 7, 14, and 28 after the operation. Each value for leakage area of FITC-albumin represents the mean percentage of total area of the ipsilateral hemisphere \pm SEM of 3 (on days 1, 3, 5, and 7) - 5 (on days 14 and 28) animals.

8. Effect of HGF treatment on leakage area of FITC albumin

The effect of HGF on the leakage of FITC-albumin on day 7 after microsphere embolism was examined. Marked degeneration and vacuolation of the right hemisphere were not seen by a gross observation of the brain and the leakage of FITC-albumin was still evident in a wide area of the brain on day 7. In addition, there was no leakage of FITC-albumin on day 14 after microsphere embolism. Therefore, the effect of HGF treatment on the leakage area of

FITC-albumin was examined on day 7 after microsphere embolism. In the ME rats, the leakage of FITC-albumin was seen in approximately 28% of the ipsilateral cerebral hemisphere, and treatment with HGF almost completely protected against it [$F(2, 9)=19.734$, $P<0.001$, post-hoc Fisher's PLSD, $P<0.001$, $n=4$ each], (Figure 3). The results indicate that the disruption of BBB induced by microsphere embolism was attenuated by HGF treatment.

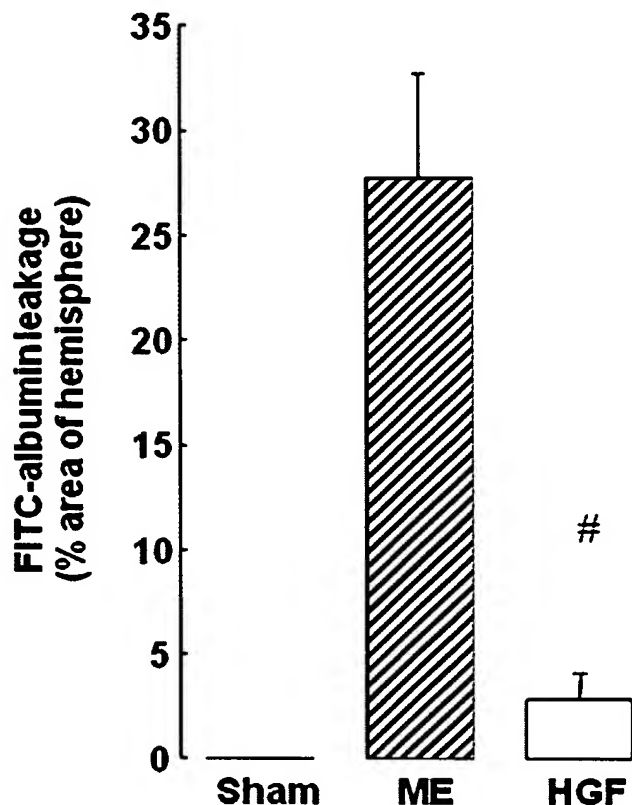


Fig. 3 Effect of HGF treatment on the leakage area of FITC-albumin in the HGF-untreated ME (ME) and 30 $\mu\text{g}/7\text{days}/\text{animal}$ HGF-treated ME (HGF) rats on day 7 after the operation. Each value for the leakage area of FITC-albumin represents the mean percentage of total area of the ipsilateral hemisphere \pm SEM of 4 animals. #Significant difference from the HGF-untreated ME group ($p<0.05$). 'Sham' indicates sham-operated animals. Leakage of FITC-albumin was measured as shown in Fig 2.

9. Effect of HGF treatment on changes in water and ion content of

cerebral hemispheres

To determine brain water and cations including Na^+ , K^+ , Ca^{2+} , and Mg^{2+} , rats were intraperitoneally anesthetized with 50 mg/kg sodium pentobarbital. Washing buffer (320 mM mannitol / 20 mM Tris HCl, pH 7.4) was transcardially perfused for 5 min at a rate of 9 mL/min by using a microtube pump, for eliminating tissue cations in vascular and extracellular spaces. A preliminary study was undertaken to determine the method for elimination of cations present in the extracellular and vascular spaces in the brain by using Cobalt-EDTA/mannitol/Tris buffer, which is believed to access vascular and extracellular, but not intracellular, spaces. Perfusion of the brain through both carotid arteries with 45 mL of the above buffer at the infusion speed of 9 mL/min was appropriate to eliminate cations present in the extracellular and vascular spaces. The right and left hemispheres of the brain were quickly isolated on ice. After the tissue had been transferred into the vial, its wet weight was determined. The tissue was dried at 120°C for 72 hrs. After estimation of dry tissue weight, the brain hemisphere was digested at 180°C with 60% (w/w) HNO_3 , and then the mixture was evaporated to dryness at 180°C. The residue was reconstituted with 0.75 N HNO_3 . The concentrations of the four cations in the supernatant fluid were determined using an atomic absorption spectrophotometer.

In the present study, water and ion content of the cerebral hemispheres were determined on day 3 after microsphere embolism, since occludin and ZO-1, tight junctional proteins of BBB (Watson et al., FASEB J 5:2013-2019, 1991), were significantly lower than the controls on day 1~ day 3 after microsphere embolism (Date et al., Neuroscience Lett 407:141-145, 2006). This result suggests the possibility that the disruption of BBB and formation of brain edema occur at an early period after microsphere embolism.

Figure 4 shows tissue water and cation content in the cerebral hemispheres of the sham-operated (Sham), HGF-untreated ME (ME), and HGF-treated ME (HGF) rats on day 3 after the operation. The brain water content in the right hemisphere of the Sham rat was not significantly altered when compared with the control, whereas that of the HGF-untreated ME rat was significantly increased when compared with the Sham animal. Treatment with HGF attenuated the increase in the brain water content of the right hemisphere of the ME rat on day 3 after the operation (ANOVA : F (3, 11)=65.386, $P<0.0001$); Fisher's PLSD: Sham vs. ME: $P<0.0001$; ME vs. HGF, $P<0.01$). A minor, but significant, decrease in tissue water content of the left hemisphere and its partial, but significant, reversal by HGF treatment were seen (ANOVA: F (3, 11)=4.402, $P<0.05$; Fisher's PLSD: Sham vs. ME, $P<0.01$; ME vs. HGF, $P<0.05$).

Changes in tissue Na^+ content of the right and left hemispheres of HGF-untreated and HGF-treated ME rats were comparable with those in water content (ANOVA: F (3, 11)=187.550, $P<0.0001$; Fisher's PLSD: Sham vs. ME, $P<0.0001$; ME vs. HGF, $P<0.05$, in the right hemisphere) and (ANOVA: F (3, 11)=5.794, $P<0.05$; Fisher's PLSD: Sham vs. ME, $P<0.01$; ME vs. HGF, $P<0.05$, in the left hemisphere).

Changes in tissue Ca^{2+} content of the right hemisphere of the ME animal on day 3 were similar to those of Na^+ content (ANOVA: F (3, 11)=204.087, $P<0.0001$; Fisher's PLSD: Sham vs. ME, $P<0.0001$; ME vs. HGF, $P<0.001$), whereas tissue Ca^{2+} content in the left hemisphere was not altered by microsphere embolism.

Although tissue K^+ and Mg^{2+} contents of the right hemisphere were decreased after microsphere embolism, these decreased contents were not affected by treatment with HGF.

The results indicate that the brain edema is induced by microsphere embolism and treatment with HGF ameliorates microsphere embolism-induced brain edema. Changes in Na^+ and Ca^{2+}

contents in the right hemisphere were synchronized with those in the brain water contents, but not those in the corresponding K^+ and Mg^{2+} content. This K^+ loss might occur due to ischemia-induced shortage of ATP production in the brain, followed by energy shortage-induced dysfunction of Na^+ , K^+ -pump and subsequent retention of K^+ in the extracellular space. Finally, the extracellular K^+ may be washed out by the perfusion with the mannitol/Tris buffer.

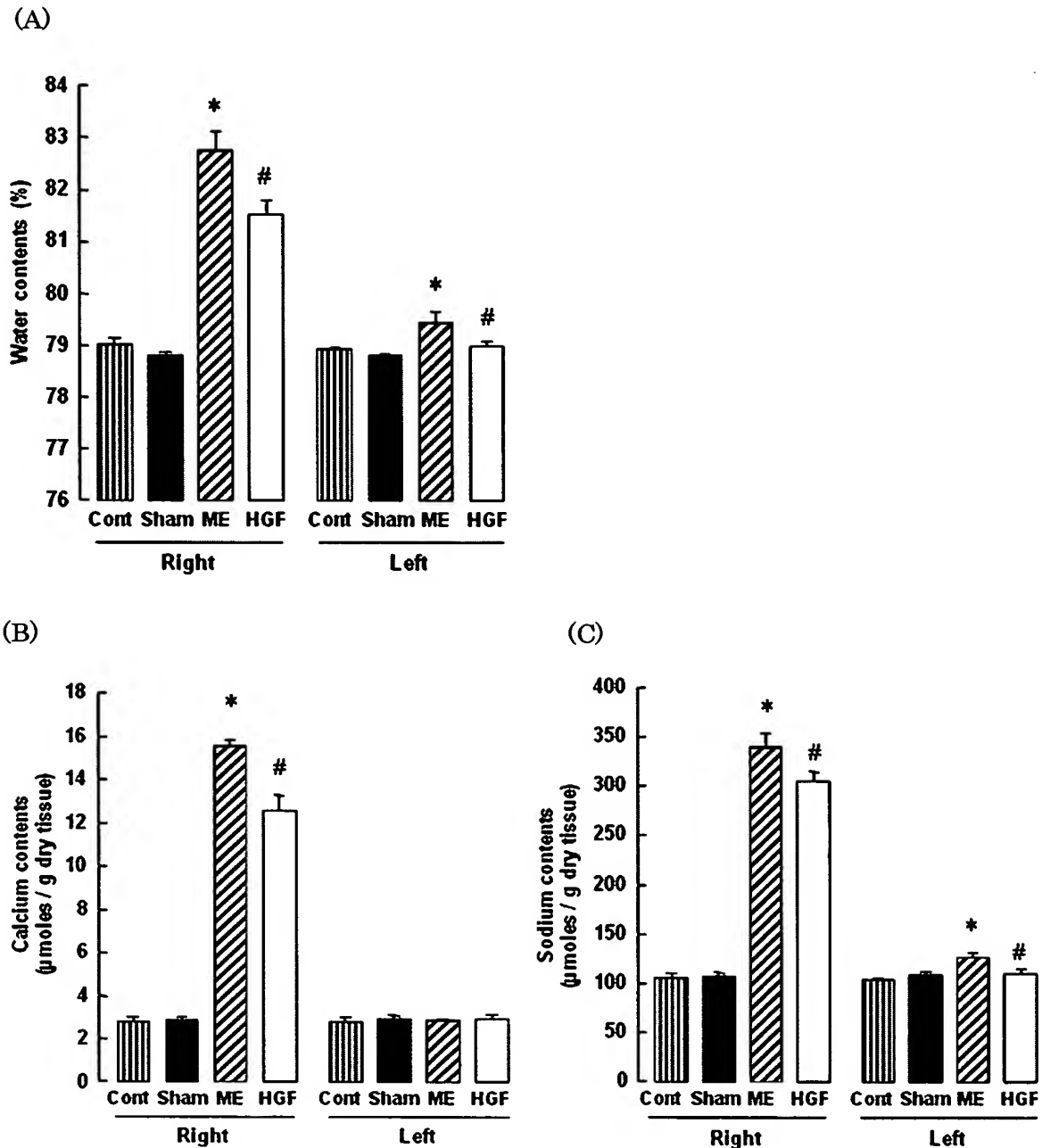


Fig. 4 Effect of HGF treatment on the brain water and cation contents in the HGF-untreated ME (ME) and 30 μ g/7days/animal HGF-treated ME (HGF) rats on day 3 after the operation. 'Cont' indicates non-operated rats. 'Sham' indicates sham-operated animals. Each value represents the mean \pm SEM of 3 (Cont) and 4 (Sham, ME, and HGF) animals. *Significant difference from the sham-operated group ($p < 0.05$); #Significant difference from the HGF-untreated ME group ($p < 0.05$). Two-way ANOVA followed by post-hoc Fisher's PLSD test revealed significant differences in changes in tissue water and cations between Sham and ME groups (*) and between ME and HGF groups (#). ($p < 0.05$).

10. Morris water maze test of the ME animal

The water maze test was performed according to the method described previously (Morris, Learn. Motiv 12:239-260, 1981; Takagi et al., Exp Brain Res 114:279-287, 1997). The water maze apparatus consisted of a circular pool with a diameter of 170 cm and a 30-cm depth of water. The water temperature was maintained at $23 \pm 1^{\circ}\text{C}$. A hidden clear circular platform with a diameter of 12 cm was placed 1.5 cm below the surface of the water and kept in a constant position in the center of 1 of the 4 quadrants of the pool. The animals were released from 3 randomly assigned start locations (excluding the platform-containing quadrant). The cut-off time for each trial was set at 180 sec. When a rat had mounted the platform, it was kept there for 30 sec. Data collection was automated by an online video-tracking device designed to track an object in a field. Escape latency (the time to climb onto the platform) and swimming speed (the distance that the animals swam divided by escape time) were determined for each trial with a behavioral tracing analyzer. Each trial was performed with an intertrial interval of approximately 1 hr. The acquisition test was started on day 12 after the operation. Animals were tested in the water maze by using a 3 trials/day regimen. The escape latency of the sham-operated rat in the acquisition test was shortened along with trial number, whereas that of the ME rat was not shortened. Treatment with HGF resulted in a shortening of the escape latency of the ME rat. Two-way ANOVA of the data in the acquisition test revealed a significant difference in the escape latency by groups [$F(2, 21) = 40.889$, $p < 0.0001$, $n = 8$ each] and by days [$F(8, 168) = 10.309$, $p < 0.0001$, $n = 8$ each].

To determine whether the animals could retain the spatial navigation ability in the hidden platform test, the retention test was performed on days 21 and 28 after the operation. The regimen and starting point used for this task were the same as those

conducted in the acquisition test on day 14. The escape latency of HGF-treated ME rats was attenuated compared with that of the HGF-untreated ME rats from the first to the third trials on days 21 and 28. Two-way ANOVA of the data in the retention test revealed a significant difference in the escape latency by groups on day 21 [$F_{2, 21}=14.530$, $p=0.0001$, $n=8$ each]. The group by day interaction was not significant ($p=0.6082$, $n=8$). On day 28 there was a significant difference in the escape latency by groups [$F(2, 21)=14.530$, $p<0.0001$, $n=8$ each]. The group by day interaction was not significant ($p=0.5227$, $n=8$). Post-hoc Fisher's PLSD test showed significant differences (*) in the escape latency at each trial in the retention test on days 21 and 28 between the Sham and ME groups (*) and between the ME and HGF groups (#) ($p<0.05$).

The swimming speed (the distance that the animals swam divided by the time to escape onto the platform) in the water maze task was similar in all groups examined as long as the swimming speed was compared with that on the same day or in the same trial order. For example, the swimming speeds on day 12 were 31.0 ± 2.1 for Sham, 25.2 ± 1.5 for ME, and 28.1 ± 3.2 cm/sec for HGF-treated ME groups ($n=8$ each).

The habituation test on day 11 and the visible platform test on day 28 were performed. There were no ME animals that must be eliminated due to a failure in habituation task and visible platform task. Thus, there were no animals to be eliminated due to the swimming disability and optical failure.

The results suggest that treatment with HGF improves learning and memory dysfunction seen after microsphere embolism.

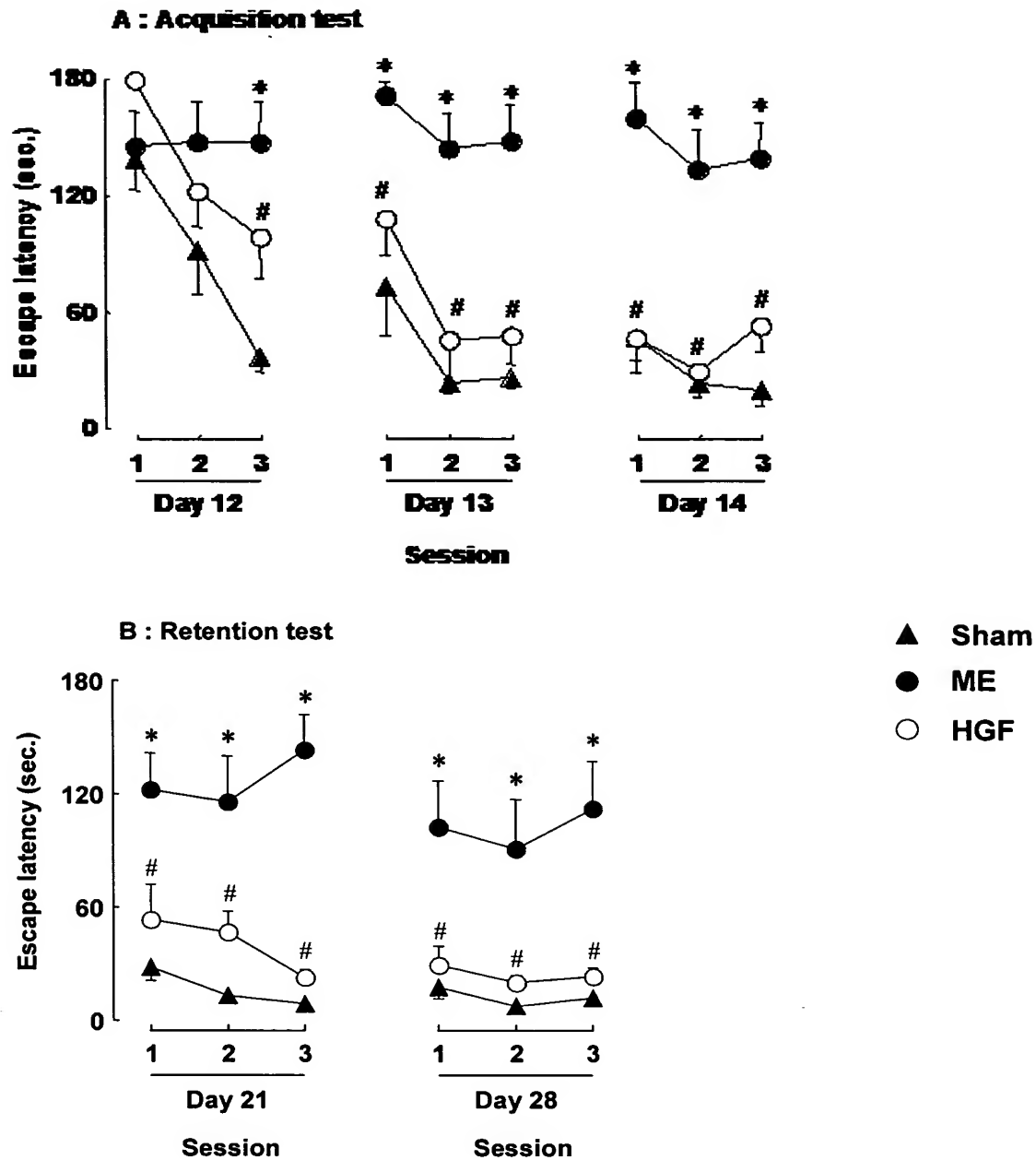


Fig. 5 The effect of HGF treatment on escape latency of the spatial learning and memory test in the water maze task.

Changes in the escape latency of the acquisition (A) and the retention (B) tests in the water maze task for the sham-operated (Sham; closed triangle), HGF-untreated ME (ME; closed circle), and 30 $\mu\text{g}/7\text{days}/\text{animal}$ HGF-treated ME (HGF, open circle) rats were determined. Each value represents the mean \pm SEM of 8 animals. There were significant differences in the escape latency between Sham and ME groups (*) and between ME and HGF groups (#) in the acquisition and retention tests.

11. Conclusion

In conclusion, the results in the current experiments suggest that HGF-treatment is capable of suppressing brain edema, which occurs at the early stage after the cerebral embolism, and contributing to the protection of the brain against cognitive deterioration following embolism-induced brain ischemia.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

This 23 day of May, 2008


Satoshi Takeo

Mediation of Systemic Vascular Hyperpermeability in Severe Psoriasis by Circulating Vascular Endothelial Growth Factor

Daniel Creamer, MBBChir, MRCP; Michael Allen, MPhil; Rhys Jaggard, PhD; Richard Stevens, MBBS, MRCP; Roy Bicknell, DPhil; Jonathan Barker, MD

Background: Severe forms of psoriasis can be complicated by systemic microvascular hyperpermeability. Vascular endothelial growth factor (VEGF) possesses potent vascular permeability activity. We suggest that VEGF enters the systemic circulation and acts on microvessels to mediate hyperpermeability.

Objectives: To quantify renal microvascular permeability and circulating VEGF concentration in severe psoriasis, and to investigate the relationship between plasma VEGF concentration and skin and joint involvement.

Design: Inception cohort studies of patients with generalized pustular psoriasis and plaque psoriasis.

Setting: St John's Institute of Dermatology, London, England.

Patients: Twenty-two patients (15 men and 7 women) with moderate and severe psoriasis were recruited (age range, 29-77 years; mean age, 47 years); 5 had generalized pustular psoriasis, 2 had erythrodermic psoriasis, and 15 had moderate-severe plaque psoriasis. An age- and sex-matched control group of 17 individuals (10 men and 7 women) was recruited (age range, 29-69 years; mean age, 42 years).

Results: There was pathological proteinuria in patients with relapsing generalized pustular psoriasis, (4-fold increase in urinary protein excretion rate in relapse compared with remission). In patients with moderate and severe psoriasis, mean plasma VEGF concentration during relapse was approximately 2.5 times greater than during remission (mean VEGF_{relapse}=257 pg/mL; mean VEGF_{remission}=103 pg/mL; $P<.01$). There was a correlation between extent of skin involvement and plasma VEGF level (mean VEGF_{severe psoriasis}=365 pg/mL; mean VEGF_{moderate psoriasis}=149 pg/mL; $P=.03$). There was a correlation between presence of psoriatic arthritis and plasma VEGF level (mean relapse VEGF_{arthritis}=277 pg/mL; mean relapse VEGF_{nonarthritis}=103.5 pg/mL; $P=.03$).

Conclusions: Generalized pustular psoriasis is accompanied by pathological proteinuria and elevated plasma VEGF levels. Plasma VEGF concentration is significantly elevated in patients with extensive skin and joint involvement and may act on renal microvasculature to induce hyperpermeability.

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From St John's Institute of Dermatology, St Thomas' Hospital, King's College Hospital, London, England (Drs Creamer and Barker and Mr Allen); the Molecular Angiogenesis Group, Imperial Cancer Research Fund, Institute of Molecular Medicine, Oxford, England (Drs Jaggard and Bicknell); and the Department of Rheumatology, St Thomas' Hospital, London (Dr Stevens).

PSORIASIS IS A common, chronic skin disease characterized by hyperproliferation of the epidermis, inflammatory cell accumulation, and elongation and exaggerated tortuosity of cutaneous blood vessels.^{1,2} Evidence^{3,4} suggests that expansion of the superficial dermal microvascular plexus in psoriasis is mediated by an active vasoproliferative process known as angiogenesis. Under physiological conditions, angiogenesis occurs in the endometrial cycle and during wound healing, whereas pathological angiogenesis is important in tumor growth and metastasis, atherosclerosis, and certain inflammatory conditions, such as rheumatoid arthritis.⁵ Vascular proliferation in angiogenesis is driven by the local expression of angiogenic factors⁶ and in psoriasis studies⁷⁻¹³ have demonstrated overexpression by lesional

skin of several angiogenic peptides, including tumor necrosis factor α , transforming growth factor α , interleukin 8, thymidine phosphorylase, endothelial cell-stimulating angiogenesis factor, angiopoietin, and vascular endothelial growth factor (VEGF).

Vascular endothelial growth factor was originally identified as a tumor cell-derived factor that induced microvascular hyperpermeability and was therefore initially termed *vascular permeability factor*.¹⁴ Subsequent studies¹⁵ characterized VEGF as an endothelial cell-specific mitogen. Vascular endothelial growth factor is recognized as a central regulator of angiogenesis because endothelial proliferation and microvascular hyperpermeability are critical early steps in the angiogenesis pathway.¹⁶ Results of clinical studies¹⁷ have suggested that high levels of circulating VEGF may induce systemic micro-

PATIENTS, MATERIALS, AND METHODS

PATIENTS

Patients participated in this study after regional and hospital ethics committee approval had been obtained. Of the 22 patients (15 men and 7 women; age range, 29-77 years; mean age, 47 years) with active psoriasis in this study, 5 had GPP, 2 had erythrodermic psoriasis, and 15 had moderate-severe plaque psoriasis (**Table 1**). Psoriasis Area and Severity Index (PASI) scoring was used to assess disease activity in patients with plaque psoriasis and in those with GPP in remission.²³ For purposes of comparison, severe psoriasis was defined as GPP or plaque disease with a PASI score greater than 30, whereas moderate psoriasis was defined as plaque disease with a PASI score less than 30. Ten of 22 patients had active psoriatic arthritis at the time of relapse of their skin disease. Patients were classified as having active arthritis if they had morning stiffness for more than 45 minutes, 5 swollen joints, and 5 tender joints.²⁴ Venous blood samples were taken from all 22 patients for VEGF analysis during relapse and remission. Blood samples were taken from premenopausal women at times outside menstruation. Urine specimens were taken from patients with GPP for protein analysis during relapse and remission. Relapse was defined as a flare of disease activity characterized by a PASI score increase greater than 70%, and remission was defined as a nadir of disease activity after treatment characterized by a PASI score decrease greater than 70%.

A control group of 17 individuals (10 men and 7 women; age range, 29-69 years; mean age, 42 years) was recruited. Venous blood samples were obtained from each control for VEGF analysis. Again, in premenopausal women, blood samples were obtained at times outside menstruation.

A separate group of 8 patients (5 men and 3 women; age range, 24-69 years; mean age, 42 years) with active psoriatic arthritis was enrolled, psoriatic arthritis being defined according to the criteria of Moll and Wright.²⁵ All patients had monoarthritis or oligoarthritis with involvement of at least 1 knee joint. Synovial fluid samples for VEGF analysis were obtained from knee joints displaying clinical signs of active synovitis (**Table 2**).

For RNA experiments, two 6-mm punch biopsy samples were taken from active plaques in 4 patients. Treatment was limited to emollients alone in the 2 weeks preceding biopsy. Normal skin tissue for RNA extraction was obtained from operative mammoplasty procedures (n=3).

URINARY PROTEIN EXCRETION

Urinary protein excretion rates (UPERs) in 5 patients with GPP were quantified in relapse and remission. Timed 24-hour samples were collected, and protein was assayed using a dye-binding colorimetric method (Biotrol Urine Proteins; Diagnostics Merck-Biotrol, Nogent-sur-Marne, France). The assay uses a molybdate-pyrogallol red complex that reacts with protein in acidic solution to form a

blue-purple complex that absorbs at 600 nm.²⁶ The color intensity measured at 600 nm is directly proportional to the protein concentration in the sample.

VEGF ENZYME-LINKED IMMUNOSORBENT ASSAY

Venous blood samples were immediately anticoagulated with sodium heparin, 10 U/mL, in sterile, endotoxin-free tubes and centrifuged at 400g for 10 minutes, supernatant removed, and stored at -70°C until required. Synovial fluid from knee effusions was drained using a sterile technique, and samples were separated, as indicated for venous blood samples, into a cellular and supernatant fraction. The 100- μ L samples of plasma and synovial fluid were immunoassayed in duplicate for human VEGF using a commercially available quantitative enzyme-linked immunosorbent assay kit that measures VEGF₁₆₅ (Quantikine; R & D Systems, Oxford, England). Although all 4 VEGF species have biological activity, VEGF₁₆₅ is soluble compared with VEGF₁₈₉ and VEGF₂₀₆, which remain cell associated and, therefore, of relevance in this study. The Quantikine kit uses a quantitative sandwich enzyme immunoassay method and has a minimum level of detection of 9 pg/mL.

RNA PREPARATION AND RIBOPROBE CONSTRUCTION

Psoriatic and normal skin specimens were homogenized using a manual microhomogenizer. The RNA was prepared using a method adapted from Chomczynski and Sacchi.²⁷ A VEGF riboprobe was designed to protect the full length of the smallest isoform (VEGF₁₂₁, yielding a 471-base band, with a lower band of 427 bases representing the remaining isoforms). This 520-base probe was generated by linearizing the full-length complementary DNA for VEGF₁₂₁ (including 26 bp of 3' untranslated sequence) cloned into pBluescript SK with EcoRV and transcribed with T7RNA polymerase.

RIBONUCLEASE PROTECTION ANALYSIS

A minimum of 100 000 cpm of each antisense riboprobe was hybridized overnight at 55°C to each sample with transfer RNA as a negative control. The RNase digestion of the unhybridized RNA fragments was achieved by adding RNase digestion buffer containing RNases A and T1 to each sample. RNases were inactivated with 12.5 μ L of a mixture containing 16% sodium dodecylsulphate solution with proteinase K, 4 μ g/ μ L. After phenol extraction and ethanol precipitation, the samples were resuspended and loaded onto 5% polyacrylamide/urea sequencing gels followed by autoradiography.²⁸ In each hybridization, an antisense transcript corresponding to human DNA topoisomerase transcribed from a construct was included as an internal control. Positive control messenger RNA from a breast carcinoma was loaded onto each gel. The resulting bands were quantitated densitometrically using a standard Gel Plotting macro and a software program (NIH Image 1.61; National Institutes of Health, Bethesda, Md). Vascular endothelial growth factor signals were normalized to the internal control (DNA topoisomerase).

vascular hyperpermeability in situations characterized by widespread capillary leak, such as the ovarian hyperstimulation syndrome. In patients with extensive, active psoriasis,

systemic disturbance is not uncommon; fever, fluid imbalance, and thermoregulatory dysfunction are recognized complications. In chronic plaque psoriasis, micro-

Table 1. Plasma VEGF Concentrations and PASI Scores in 22 Patients With Psoriasis During Relapse and Remission*

Patient No.	Psoriasis Type	Arthritis	Relapse		Remission	
			PASI Score	VEGF, pg/mL	PASI Score	VEGF, pg/mL
1	GPP	-	...	1003.5	14.4	545.3
2	GPP	+	...	472.7	10.0	133.2
3	GPP	-	...	525.5	11.2	19.8
4	GPP	+	...	391.9	3.3	174.5
5	GPP	+	...	194	8.2	98.5
6	EdP	-	42.0	121.1	8.4	36.8
7	EdP	+	31.8	96.8	8.8	8.0
8	PIP	+	42.2	470	7.6	192
9	PIP	+	42.0	215	4.3	131.5
10	PIP	+	35.3	308.7	3.7	82.5
11	PIP	-	33.0	211.4	5.0	55.3
12	PIP	-	28.7	45	3.3	52.6
13	PIP	+	27.8	536	4.3	133.4
14	PIP	+	27.0	235.1	2.4	94.1
15	PIP	-	25.1	92.6	3.2	64.7
16	PIP	-	20.4	86.1	8.3	119.1
17	PIP	+	18.9	159.6	7.7	88.1
18	PIP	-	17.8	67.6	4.3	86.1
19	PIP	-	17.6	206.8	1.9	74.8
20	PIP	-	16.7	73.1	2.4	14.9
21	PIP	-	14.0	53.2	1.6	9.9
22	PIP	-	12.6	78.1	1.3	45.9

*VEGF indicates vascular endothelial growth factor; PASI, Psoriasis Area and Severity Index; GPP, generalized pustular psoriasis; -, no arthritis; ellipses, not applicable; +, active arthritis present; EdP, erythrodermic psoriasis; and PIP, plaque psoriasis.

albuminuria indicates subclinical renal microvascular hyperpermeability,¹⁸ whereas further studies¹⁹ have demonstrated that the extent of albuminuria reflects the degree of psoriatic skin involvement. Microalbuminuria in psoriasis may result from the activity of a circulating permeability factor produced by lesional tissue. Following our reported²⁰ observation of elevated plasma VEGF in erythrodermic psoriasis, we hypothesize that in severe psoriasis, VEGF, elaborated by lesional psoriatic tissue, enters the systemic circulation and acts in an endocrine fashion on renal microvasculature to induce clinically significant hyperpermeability.

There are several reports^{21,22} of severe pulmonary edema occurring in patients with generalized pustular psoriasis (GPP), the edema accumulating as a direct consequence of increased pulmonary microvascular permeability. Hypoalbuminemia is a common complication of GPP, and this association may again reflect microvascular hyperpermeability with protein loss into the gastrointestinal or renal tracts. In the present study, renal microvascular permeability and circulating VEGF have been quantified in patients with GPP during relapse and remission. In a larger group of patients with moderate and severe psoriasis, correlation has been sought between the extent of skin involvement and plasma VEGF concentration and between the presence of psoriatic arthritis and plasma VEGF concentration. Lesional skin and joint fluid has been assayed to identify the source of circulating VEGF in psoriasis.

RESULTS

URINARY PROTEIN EXCRETION RATES

All 5 patients with GPP demonstrated pathological UPERs during relapse (range, 0.15-1.59 g/24 h; mean,

Table 2. Clinical Details and Synovial VEGF Concentrations in 8 Patients With Psoriatic Arthritis*

Patient No.	Arthritis	Synovial VEGF, pg/mL
1	Oligoarthritis	2528.6
2	Oligoarthritis	2411.1
3	Monoarthritis	3284.5
4	Oligoarthritis	463.8
5	Oligoarthritis	1258.6
6	Oligoarthritis	1225.0
7	Oligoarthritis	2614.4
8	Oligoarthritis	1990.2

*VEGF indicates vascular endothelial growth factor.

0.55 g/24 h; reference value, <0.15 g/24 h). During remission, 4 of 5 UPER values returned to within reference values (range, 0.09-0.20 g/24 h; mean, 0.14 g/24 h; reference value, <0.15 g/24 h) (**Figure 1**). There was a mean 4-fold increase in UPER in relapse compared with remission.

PLASMA VEGF ANALYSIS

The 5 patients with GPP demonstrated mean plasma VEGF levels 2.6-fold greater in relapse compared with remission (**Figure 2**). In the larger group of 22 patients (5 with GPP, 2 with erythrodermic psoriasis, and 15 with moderate-severe plaque psoriasis), mean plasma VEGF_{relapse} concentration was approximately 2.5 times greater than VEGF_{remission} (mean \pm SEM VEGF_{relapse} = 257 \pm 49 pg/mL and VEGF_{remission} = 103 \pm 6.7 pg/mL; $P < .01$, 2-sample t test) (**Figure 3**). Plasma VEGF concentration in an age- and sex-matched control group ($n = 17$) was significantly lower than VEGF_{relapse} and VEGF_{remission} (mean \pm SEM

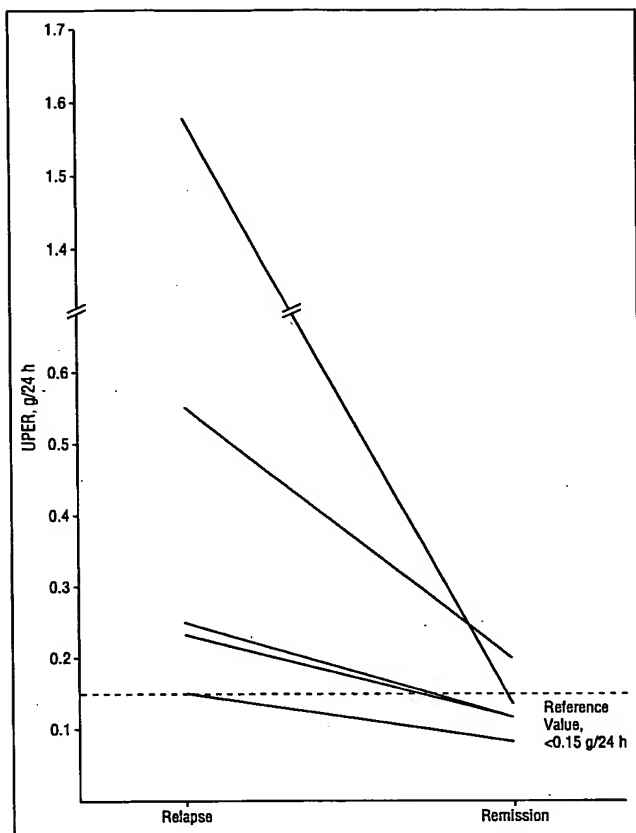


Figure 1. Urinary protein excretion rate (UPER) in 5 patients with generalized pustular psoriasis during relapse and remission.

$VEGF_{\text{remission}} = 103 \pm 6.7$ pg/mL, $VEGF_{\text{control}} = 24.7 \pm 6.7$ pg/mL; $P < .001$, 2-sample t test) (Figure 3).

Comparison of plasma VEGF levels in patients with severe psoriasis (GPP+PASI score >30) ($n=11$) vs those with moderate psoriasis (PASI score <30) ($n=11$) demonstrated significantly higher VEGF levels in the severe group (mean \pm SEM $VEGF_{\text{severe}} = 365 \pm 78$ pg/mL, $VEGF_{\text{moderate}} = 149 \pm 43$ pg/mL; $P = .03$, 2-sample t test) (Figure 4).

A relationship was demonstrated between circulating VEGF levels and the presence of active psoriatic arthritis (mean \pm SEM relapse $VEGF_{\text{arthritis}} = 277 \pm 53$ pg/mL [$n=10$], mean relapse $VEGF_{\text{nonarthritis}} = 103.5 \pm 19.0$ pg/mL [$n=12$]; $P = .03$, 2-sample t test) (Figure 5).

PSORIATIC SYNOVIAL FLUID VEGF ANALYSIS

In another group of 8 patients with active psoriatic arthritis, synovial fluid VEGF was assayed using enzyme-linked immunosorbent assay (Table 2). The VEGF enzyme-linked immunosorbent assay has a minimum level of detection for $VEGF_{165}$ of 9 pg/mL. High synovial VEGF concentrations (mean \pm SEM, 1972 ± 221.1 pg/mL) were identified in each case.

RIBONUCLEASE PROTECTION ANALYSIS

Ribonuclease protection assays for VEGF are shown in Figure 6. Short (48-hour) exposure demonstrated a

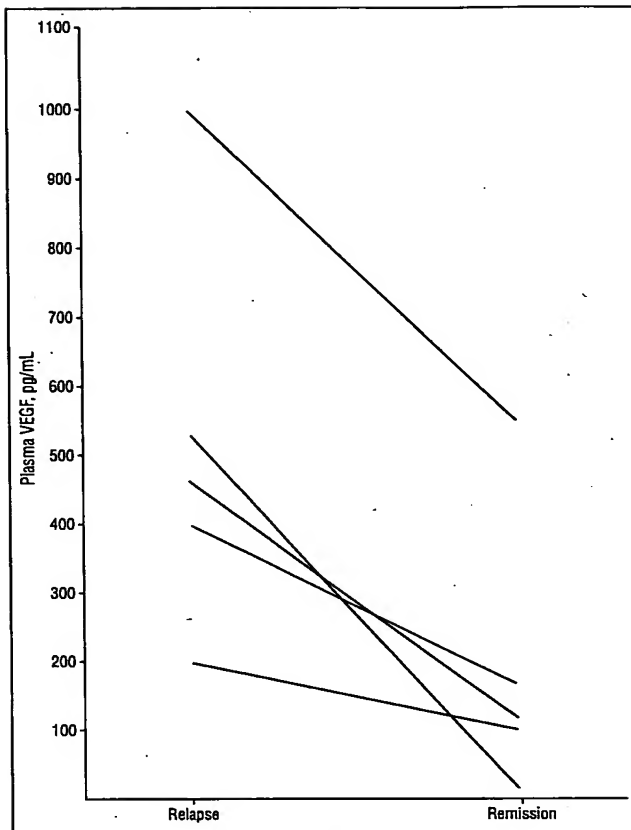


Figure 2. Plasma vascular endothelial growth factor (VEGF) concentration in 5 patients with generalized pustular psoriasis during relapse and remission.

strong signal for VEGF in all 4 psoriasis samples. In normal skin ($n=3$) at the same exposure, VEGF signals are of low intensity. To obtain values of fold-change in messenger RNA levels, messenger RNA abundance was quantitated from autoradiographic data by scanning laser densitometry. Signals from the VEGF messenger RNA were normalized to those of the internal topoisomerase control. Quantification by this method showed an approximate 4-fold increase in VEGF signal in lesional vs normal skin.

COMMENT

Our findings demonstrate that GPP is accompanied by pathological proteinuria and markedly elevated concentrations of plasma VEGF. During remission, urinary protein excretion normalizes and circulating VEGF levels return to control values. In a larger group of patients (including those with GPP, erythrodermic psoriasis, and plaque psoriasis), plasma VEGF concentration is consistently higher in relapse than in remission and is significantly elevated in patients with extensive skin involvement and active joint disease. We suggest that VEGF, synthesized in psoriatic skin and synovium, enters the systemic circulation and may act on renal microvasculature to induce hyperpermeability with consequent proteinuria.

Renal microvascular hyperpermeability permits the escape of larger protein molecules and those of smaller mo-

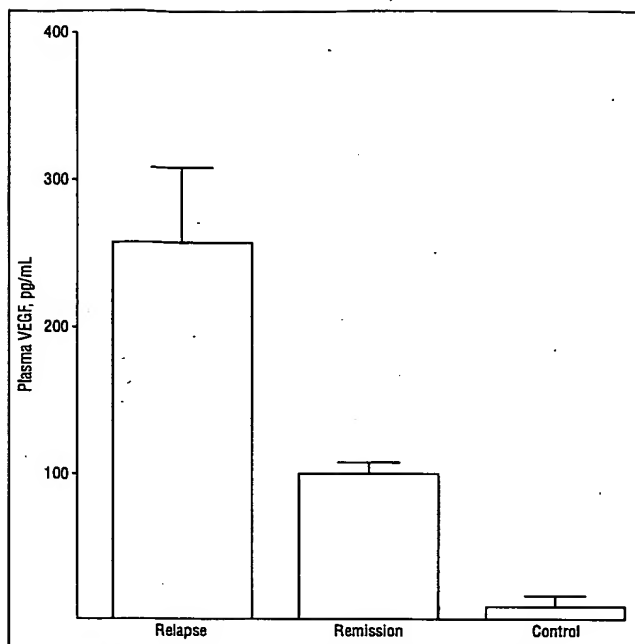


Figure 3. Mean plasma vascular endothelial growth factor (VEGF) concentration in 22 patients with psoriasis (5 with generalized pustular psoriasis, 2 with erythrodermic psoriasis, and 15 with moderate-severe plaque psoriasis) during relapse and remission vs 17 age- and sex-matched controls. Error bars represent SEM.

lecular weight (eg, albumin), which pass into the glomerular filtrate and are clinically measurable as proteinuria. Microalbuminuria, defined as mildly elevated levels of proteinuria (30-200 mg/L), has been reported in patients with mild-moderate psoriasis by Cecchi et al.¹⁹ The mean UPER in their cohort of patients with a PASI score greater than 11 was 28.8 mg/24 h, whereas the mean relapse UPER in our GPP group was 560 mg/24 h, which reduced to 140 mg/24 h during remission (reference value, <150 mg/24 h). The results of Cecchi and colleagues and our own data suggest the presence of renal microvascular hyperpermeability in psoriasis that increases with intensity of skin disease but reverses with successful treatment.

Within the papillary dermis of lesional, psoriatic skin, the superficial microvasculature is characterized by an angiogenic and hyperpermeable phenotype, features that contribute to the development and persistence of skin lesions in psoriasis. Microvascular hyperpermeability at any site can be mediated by several biologically active substances, including VEGF, which has permeability activity 40 000 times greater than histamine on a molar basis.²⁹ Detmar et al¹³ initially identified VEGF overproduction in psoriatic epidermis, and they³⁰ subsequently demonstrated the central role of keratinocyte-derived VEGF in changes to underlying superficial dermal microvasculature. In addition to acting locally to induce angiogenesis and microvascular hyperpermeability, circulating VEGF has been implicated in systemic capillary permeability associated with conditions such as ovarian hyperstimulation syndrome and tumor ascites.^{17,31} Other studies have demonstrated up-regulation of VEGF in pathological conditions char-

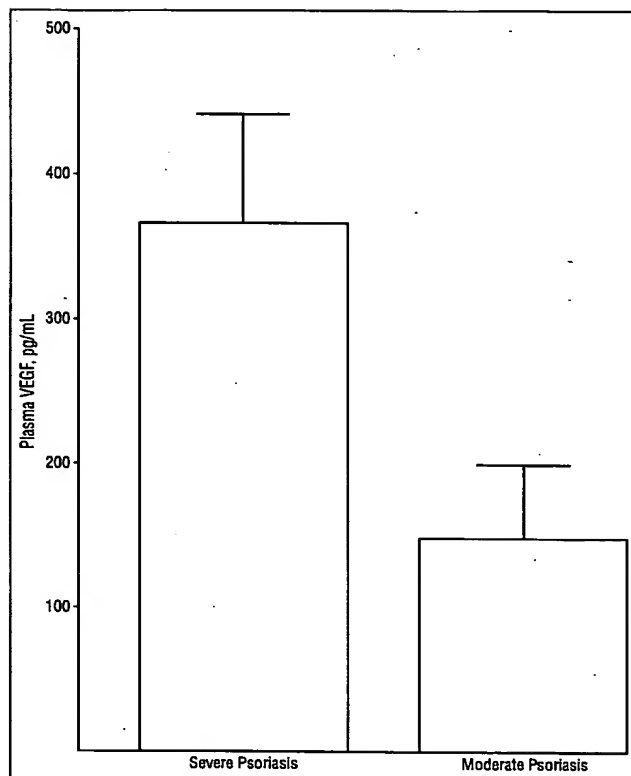


Figure 4. Comparison of mean plasma vascular endothelial growth factor (VEGF) levels in patients with severe psoriasis (generalized pustular psoriasis and Psoriasis Area and Severity Index [PASI] score >30) (n=11) vs patients with moderate psoriasis (PASI score <30) (n=11). Error bars represent SEM.

acterized by proteinuria and increased renal microvascular permeability.³²

Bhushan et al¹¹ reported an association among VEGF concentration in lesional skin, extent of psoriatic skin involvement (PASI), and VEGF concentration in peripheral blood. In our experiments, the finding that patients with severe psoriasis (GPP + PASI score >30) had significantly higher levels of plasma VEGF compared with patients with moderate psoriasis (PASI score <30) again suggests that circulating levels of VEGF reflect the extent of psoriatic skin involvement. Further evaluation revealed that patients with active psoriatic arthritis had significantly higher levels of circulating VEGF than those without arthropathy, whereas separate experiments demonstrated high concentrations of VEGF in the articular fluid of involved psoriatic joints. High synovial VEGF concentrations in psoriatic synovial fluid were initially reported by Fearon et al,³³ and our results are consistent with their data. These findings indicate that an articular source may contribute, along with the cutaneous source, to circulating VEGF concentration in patients with active psoriasis.

We hypothesize that there may be a causal relationship between renal microvascular hyperpermeability in patients with severe psoriasis and high circulating VEGF levels. Reports of pulmonary edema in GPP secondary to the capillary leak syndrome suggest the involvement of pulmonary microvascular hyperpermeability, which may be mediated by a circulating vasoactive cytokine, such as VEGF.^{16,17} Although the renal and pulmonary vasculature can respond

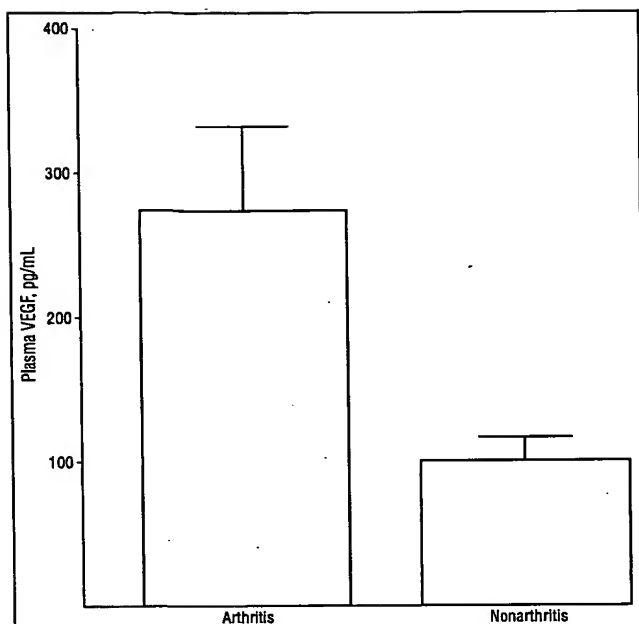


Figure 5. Comparison of mean plasma vascular endothelial growth factor (VEGF) levels in patients with psoriasis and active arthritis (n=10) vs those with psoriasis without arthritis (n=12) during disease relapse. Error bars represent SEM.

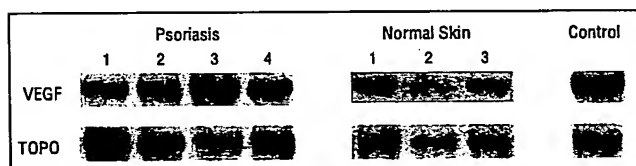


Figure 6. Ribonuclease protection assays for vascular endothelial growth factor (VEGF) messenger RNA from lesional psoriatic skin (n=4) and normal nonpsoriatic skin (n=3). Control messenger RNA was from a breast carcinoma sample. The internal control is DNA topoisomerase (TOPO).

to circulating permeability signals in severe psoriasis, other microvascular beds seem to be resistant to systemic hyperpermeability factors. Organ-dependent variations in response to VEGF may be explained by a lack of accessibility of bioactive VEGF in certain sites or because of qualitative or quantitative differences in VEGF receptors.

Plasma VEGF analysis in patients with severe psoriasis may be a useful predictor of clinical outcome and affect management. In addition, VEGF and VEGF-mediated pathways may represent potential targets in the development of future therapeutic strategies in psoriasis.

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Corresponding author and reprints: Daniel Creamer, MB, BChir, MRCP, Department of Dermatology, King's College Hospital, Denmark Hill, London SE5 9RS, England (e-mail: daniel.creamer@kingshc.nhs.uk).

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Reference 2

(12) **United States Patent**
Nakamura

(10) Patent No.: **US 6,699,837 B2**
(45) Date of Patent: ***Mar. 2, 2004**

(54) **TREATMENT OF NEURONS WITH HGF**

(76) Inventor: **Toshikazu Nakamura**, 10-27,
Takamidai, Takatsuki-shi, Osaka 569
(JP) *filed as CONT*

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Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days. *9/16/2014*

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Primary Examiner—Gary Kunz

Assistant Examiner—Robert C. Hayes

(74) Attorney, Agent, or Firm—Birch, Stewart, Kolasch & Birch, LLP

(57) **ABSTRACT**

The invention relates to a therapeutic agent for disorder in brain and nerve containing HGF (hepatocyte growth factor) as an active ingredient, and a method for treatment of disorder in brain and nerve comprising administration of HGF. The active ingredient HGF possesses an action to prolong survival of brain and nerve cells, and the injured brain or nerves may be regenerated and restored. Therefore, the therapeutic agent and method for treatment of the invention are useful for prevention and treatment of various disorder in brain and nerve (for example, dementia, senile dementia of Alzheimer type, cerebral stroke, and cerebral infarction).

3 Claims, 8 Drawing Sheets

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-continued

690	695	700
Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile Ile Leu Thr Tyr Lys Val		
705	710	715
Pro Gln Ser		

What is claimed is:

1. A method of promoting survival of neurons following cerebral stroke/ischemia or promoting survival of hippocampal or olfactory bulb neurons of a human or a mammal, which comprises administering an effective amount of human HGF protein comprising SEQ ID NO: 2 or SEQ ID NO:4.

2. A method of prolonging the survival of neurons following cerebral stroke/ischemia or prolonging the survival of hippocampal or olfactory bulb neurons comprising con-

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tacting the neurons with an effective amount of human HGF protein comprising SEQ ID NO: 2 or SEQ ID NO:4.

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3. A method of prolonging survival of hippocampal or olfactory bulb neurons during cerebral stroke and cerebral infarction, which comprises administering an effective amount of human HGF protein comprising SEQ ID NO: 2 or SEQ ID NO: 4.

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